ENZYMES OF ANTARCTIC FISHES: EFFECT OF TEMPERATURE ON CATALYSIS *

by

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ABSTRACT. - We have investigated the temperature effect on the catalytic activity of three enzymes from Antarctic fish: glucose-6-phosphate dehydrogenase (G6PD) from the blood of the white-blooded channichthyid *Chionodraco hamatus* and the red-blooded nototheniid *Dissostichus mawsoni*. L-glutamate dehydrogenase (GDH) from the liver of the channichthyid *Chaenocephalus aceratus* and glycogen phosphorylase *b* from the muscle of the nototheniid *Trematomus bernacchii*. The study of the temperature effect on the catalytic activity of G6PD and GDH shows that the Antarctic enzymes are more efficient catalysts at low temperature than the mesophilic homologous enzymes. The activity of Antarctic G6PD increases at increasing temperatures; above 55°C irreversible heat inactivation occurs. A different behavior is shown by *C. aceratus* GDH. This enzyme has an apparent optimal temperature at 25°C; above this temperature, the catalytic activity decreases and irreversible heat inactivation is then observed above 45°C. All three enzymes are irreversibly inactivated at temperatures much higher than the physiological ones and only slightly lower than those of the homologous mesophilic enzymes. In Antarctic phosphorylase *b*, the allosteric activator AMP protects against inactivation. In general, heat inactivation of these enzymes occurs very abruptly in a narrow temperature range.

RÉSUMÉ. - Enzymes des Poissons antarctiques: influence de la température sur la catalyse.

Nous avons recherché quelle est l'influence de la température sur l'activité catalytique de trois enzymes chez des poissons antarctiques: la glucose-6-phosphate deshydrogénase (G6PD) extraite du sang du poisson à sang incolore, le Channichthyidae *Chionodraco hamatus*, et du poisson à sang normal, le Nototheniidae *Dissostichus mawsoni*; la L-glutamate deshydrogénase (GDH) à partir du foie du Channichthyidae *Chaenocephalus aceratus*; et la phosphorylase *b* provenant du muscle du Nototheniidae *Trematomus bernacchii*. L'étude de l'influence de la température sur l'activité catalytique de la G6PD et de la GDH montre que les enzymes antarctiques sont des catalyseurs plus efficaces à basse température que les enzymes homologues mésophiles. L'activité de la G6PD antarctique s'accroît lorsque la température augmente; au-dessus de 55°C survient une inactivation irréversible. Il en est autrement avec la GDH de *C. aceratus*. Cette enzyme présente une activité optimale à 25°C; au-dessus de cette température, l'activité catalytique décroît et une inactivation irréversible est observée au-dessus de 45°C. Ces trois enzymes sont irréversiblement inactivées à des températures bien supérieures aux températures physiologiques et seulement à des températures légèrement plus basses que les enzymes homologues mésophiles. En ce qui concerne la phosphorylase *b* antarctique, l'activateur allostérique AMP la protège de l'inactivation. En général, l'inactivation par la chaleur de ces enzymes intervient brutalement dans une gamme de température très étroite.

Key-words. - Nototheniidae, Channichthyidae, PSE, Antarctic Ocean, Enzyme, Temperature effect.

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Antarctic fish live in an extreme environment at the constant temperature of -1.97°C throughout the year. The cellular environment of ectothermic species such as fish is characterized by the same temperature. It is well known that environmental factors, such as temperature, have profound effects on the catalytic activity and on enzyme regulation and structure. At decreasing temperatures the rates of enzyme-catalyzed reactions are reduced by the low heat content of the cellular environment (Q_{10} effect) that affects the higher orders of protein structure and the interactions of the protein with low-molecular-weight ligands, and therefore the formation of the enzyme-substrate complex. The strategies of biochemical adaptation developed during the cold-adaptation process allow low-body-temperature species to obtain an adequate metabolic flux, offsetting the Q_{10} effect.

The study of enzymes from cold-adapted fish is still too scarce to allow the understanding of the molecular properties acquired during adaptation. A wider literature on cold-adapted enzymes from psychrophilic bacteria is available. Comparison with enzymes from bacteria or with secretory enzymes is useful, but must be taken with some caution. In fact, Antarctic fish are stenothermic organisms, whereas bacteria usually tolerate a wider temperature range. Furthermore, some of the cold-adapted enzymes so far studied are secretory proteases (Genicot *et al.*, 1988; Feller *et al.*, 1994) that could have developed particular adaptations linked to specific structural and functional requirements. Therefore, in order to identify trends linked to cold-adaptation of Antarctic fish, it is probably more correct to compare intracellular enzymes from stenothermic organisms.

Under the conditions of the habitat, a higher catalytic efficiency than that of homologous enzymes from temperate organisms is a property shown by several enzymes from Antarctic fish (Hochachka and Somero, 1984; Mcdonald *et al.*, 1987; Somero, 1991; di Prisco *et al.*, 1994), shared with enzymes from psychrophilic bacteria (Davail *et al.*, 1994; Feller *et al.*, 1994). For example, trypsin from the Antarctic fish *Paranotothenia magellanica* shows a higher catalytic efficiency than that of trypsin from trout and ox, over the whole range of the investigated temperature (Genicot *et al.*, 1988); pyruvate kinase from *Trematomus bernacchii* catalyses the reaction with a lower activation energy than the enzyme from trout (Somero and Hochachka, 1968); lactate dehydrogenase activity is high in the heart of the icefish *Channichthys rhinoceratus*, due to the increase of both enzyme concentration and specific activity (Feller *et al.*, 1991).

The higher catalytic efficiency shown by several cold-adapted enzymes is generally associated with a more flexible structure, which allows conformational changes during catalysis to occur with less energy input (Hochachka and Somero, 1984; Gudmundsdóttir *et al.*, 1994; Feller *et al.*, 1994). Therefore the question arises whether the more flexible structure of Antarctic enzymes necessarily implies lower heat stability.

Knowledge of thermostability of enzymes from Antarctic fish does not yet allow to identify a clear trend, since the available information is still scanty. Feeney and Osuga (1971) studied the thermostability of different enzymes from Antarctic fish muscle in comparison with that shown by homologous mesophilic enzymes. They reported that the temperatures of half inactivation of glycogen phosphorylase from *Dissostichus mawsoni* and *Pagothenia borchgrevinki* did not substantially differ from those of the enzyme from rabbit, trout and salmon, the range being from 42°C to 48°C. These authors reported that glyceraldehyde-3-phosphate dehydrogenase from *D. mawsoni* and rabbit had nearly identical temperature stability curves; on the contrary, the half-inactivation temperatures of *P. borchgrevinki*, *D. mawsoni* and rabbit muscle fructose-1,6-diphosphate aldolases were 29°C, 38°C and 55°C respectively, indicating a lower thermostability of the Antarctic enzymes. More recently, it was reported that some enzymes from cold-adapted organisms

such as trypsin from *Paranotothenia magellanica* (Genicot *et al.*, 1988), lipases from psychrotrophic *Moraxella* strains from Antarctica (Feller *et al.*, 1990) and alkaline phosphatase from *Sphingobacterium antarcticus* (Chattopadhyay *et al.*, 1995), are more sensitive to temperature than the mesophilic counterparts.

In order to investigate the structure-function relationships in enzymes from cold-adapted fish we have studied the temperature effect on the catalytic activity and thermostability of three enzymes from Antarctic fish: glucose-6-phosphate dehydrogenase (G6PD) from the blood of the white-blooded icefish *C. hamatus* and the red-blooded notothenid *D. mawsoni*; L-glutamate dehydrogenase (GDH) from the liver of the icefish *C. aceratus* and glycogen phosphorylase *b* from the muscle of the notothenid *T. bernacchii*.

MATERIALS AND METHODS

Materials

Specimens of *Chionodraco hamatus* and *Trematomus bernacchii* were collected by gill nets in Terra Nova Bay, Ross Sea, Antarctica, and kept at Terra Nova Bay Station (74° 42'S, 164°07'E), in aquaria supplied with running sea water, at approximately 0°C. Specimens of *Chaenocephalus aceratus,* collected by bottom trawling in Dallman Bay and Low Island, were from Palmer Station, Antarctica (64°46'S, 64°03'W). Blood was drawn from the caudal vein of unanaesthetised fish, by means of heparinised syringes. Red cells were immediately separated by low speed centrifugation in the cold and washed with a solution of 1.7% NaCl in 1 mM Tris-HCl, pH 8.1. The small white sediment of channichthyid blood was used without saline washing, in view of the fragility of the erythrocyte-like cells. Freshly drawn blood of *Dissostichus mawsoni* was kindly provided by A.L. DeVries at McMurdo Station.

Enzyme assay

Standard assay of G6PD activity was carried out at 20°C by recording the reduction of $NADP^+$ at 340 nm in a Varian DMS 300 thermostated spectrophotometer. The standard assay mixture contained 100 mM Tris-HCl pH 7.6, 0.2 mM $NADP^+$ and 1 mM G6P. The reaction was initiated by the addition of 0.1-0.2 µg of enzyme in a final volume of 1 ml.

Standard GDH activity assay was carried out at 20°C by recording the oxidation of NADH (reverse reaction) at 340 nm. The assay mixture for α-ketoglutarate reductive amination contained 100 mM potassium phosphate buffer pH 7.5, 100 mM ammonium sulfate, 2 mM α-ketoglutarate, 40 mM NADH and 0.2-0.4 µg of enzyme in a final volume of 1 ml.

A coupled assay system (Helmreich and Cori, 1964) was used to detect the catalytic activity of glycogen phosphorylase *b* by recording the reduction of $NADP^+$ at 340 nm. The standard assay mixture contained 45 mM Tris-acetate, 2 mM EDTA-Na_2, 10 mM/µg acetate, 1 mM 2-mercaptoethanol, 0.6 mM $NADP^+$, 0.1% glycogen, 0.5 mM AMP, 10 mM potassium phosphate, 10 µg/ml phosphoglucomutase and 4 µg/ml glucose-6-phosphate dehydrogenase. The reaction was initiated by addition of 0.5-1 µg of enzyme in a final volume of 1 ml.

One enzymatic unit is defined as the amount of enzyme catalyzing the reaction of 1 µmole of substrate/min under the standard assay conditions. All assays were run in triplicate.

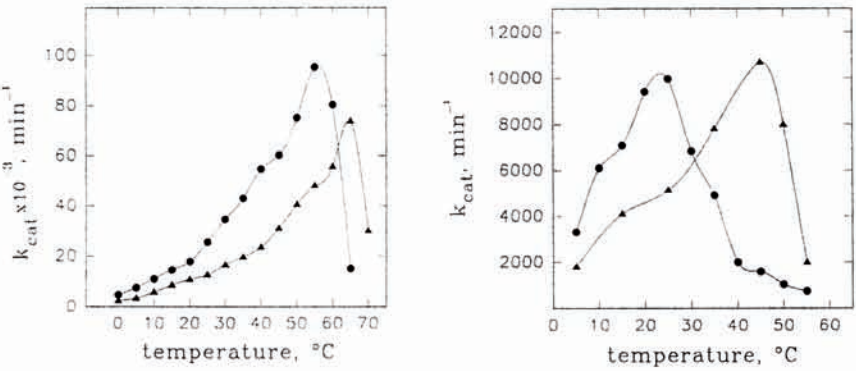

Fig. 1. - Temperature effect on the catalytic activity of *D. mawsoni* (●) and human (▲) G6PDs.
Fig. 2. - Temperature effect on the catalytic activity of *C. aceratus* (●) and bovine (▲) GDHs (reverse reaction).

Thermostability

Heat inactivation experiments were performed incubating the enzyme at the given temperatures; at time intervals aliquots were taken and assayed in standard conditions at 20°C. Half inactivation temperatures were calculated after incubation for 10 min. at the selected temperatures.

RESULTS

Temperature effect on the catalytic activity

Glucose-6-phosphate dehydrogenase

The effect of temperature on the catalytic activity of G6PD, purified from the erythrocytes of the red-blooded notothenid *Dissostichus mawsoni* and from the blood of the icefish *Chionodraco hamatus* (Ciardiello *et al.*, 1995) has been investigated in the range 0-70°C. Figure 1 shows the results obtained in comparison with G6PD purified from human erythrocytes (Descalzi-Cancedda *et al.*, 1984); the enzyme from *C. hamatus* displayed a behavior similar to that of *D. mawsoni* G6PD (data not shown). Both at 0°C (near the physiological temperature) and at higher temperature, *D. mawsoni* G6PD displayed a catalytic activity approximately two-fold higher than that measured in the human enzyme. The apparent optimum temperature shown by *D. mawsoni* and human G6PD was 55°C and 65°C, respectively; above these temperatures fast and irreversible heat inactivation occurred.

L-Glutamate dehydrogenase

The temperature effect on the catalytic activity of GDH, purified from the liver of the icefish *C. aceratus*, has been studied in the temperature range 5-60°C in comparison with the homologous enzyme from ox liver. At temperatures lower than 30°C, the catalytic rate of *C. aceratus* GDH was higher than that of the mesophilic enzyme (Fig. 2). The latter showed an apparent optimum temperature at 45°C (above which irreversible heat inactivation occurred), whereas the apparent optimum temperature of the Antarctic enzyme was at 25°C; above this temperature the catalytic rate decreased, although the inactivation was fully reversible, becoming irreversible only above 45°C (as shown by the thermostability experiments below).

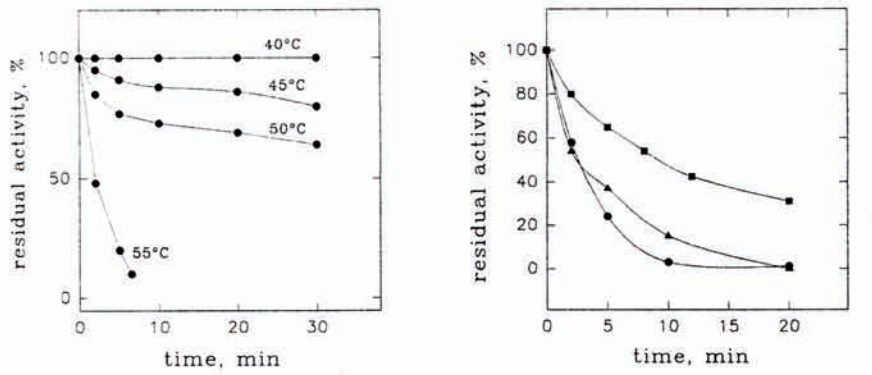

Fig. 3. - Time course of *Dissostichus mawsoni* G6PD heat inactivation. The incubation mixture was 50 mM potassium phosphate buffer pH 7.5, 25 mM NaCl, 1 mM EDTA, 0.2% 2-mercaptoethanol; the enzyme concentration was 0.05 mg/ml.

Fig. 4. - Time course of heat inactivation at 55°C of G6PD from *D. mawsoni* (●), *Chionodraco hamatus* (▲) and human (■). The incubation mixture was as in Fig. 3.

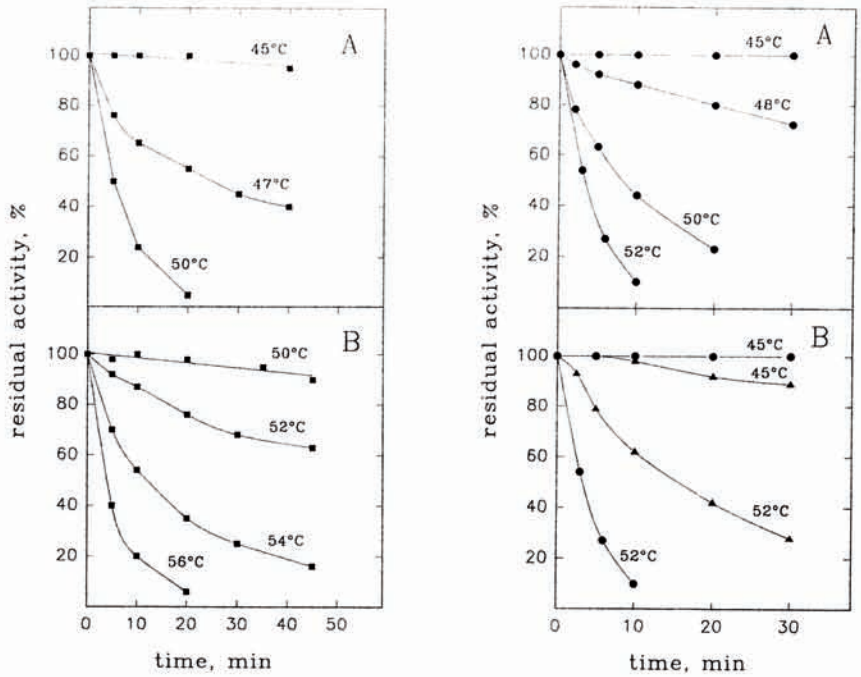

Fig. 5. - Time course of *Trematomus bernacchii* glycogen phosphorylase *b* heat inactivation in the absence (A) and presence (B) of 1 mM AMP. The enzyme (0.2 mg/ml) was incubated in 25 mM Tris-acetate, pH 7.5.

Fig. 6. - Time course of *Chaenocephalus aceratus* (●, panels A and B) and bovine (▲, panel B) GDH heat inactivation. The enzyme (0.15 mg/ml) was incubated in 100 mM potassium phosphate buffer, pH 7.5.

Thermostability

Glucose-6-phosphate dehydrogenase

Figure 3 shows the time course of heat inactivation of G6PD purified from the notothenid *D. mawsoni*; the behavior of G6PD from the icefish *C. hamatus* was similar. The heat response of Antarctic G6PDs was similar to that of the human enzyme at temperatures up to 50°C, with retention of full activity after 30 min. of incubation at 40°C, and slow inactivation at 45°C and 50°C. Very fast inactivation occurred at 55°C, where a substantial difference was observed, the Antarctic enzymes being inactivated at much higher rate than the human enzyme (Fig. 4). Thus, a small temperature increase produced a large effect only on the Antarctic enzyme activity.

The temperatures of half inactivation, after 10 min. of incubation, were similar, i.e. 52°C for Antarctic G6PDs and 55°C for the human enzyme.

Glycogen phosphorylase b

The time course of heat inactivation of phosphorylase *b*, purified almost to homogeneity from the muscle of the notothenid *T. bernacchii*, is shown in figure 5A. Again, a strong effect of temperature on the activity was observed in a narrow temperature range (45-50°C). The allosteric activator AMP protected the enzyme (Fig 5B), shifting the curves of heat inactivation of 5-6°C towards higher temperatures. The most striking effect was observed at 50°C, where the enzyme retained almost 100% activity after 20 min. incubation in the presence of the ligand, but was completely inactivated in its absence.

Phosphorylase *b* displayed a high temperature of half inactivation (49°C in the absence and 55°C in the presence of AMP).

L-Glutamate dehydrogenase

The time course of heat inactivation of GDH from *C. aceratus* liver at several temperatures is shown in figure 6A. The loss of activity of Antarctic GDH, induced by high temperature, was compared with that shown by the bovine enzyme (Fig. 6B). Both enzymes were heat inactivated at temperatures higher than 45°C. The data of Fig. 6B illustrate that also this Antarctic enzyme was fully heat inactivated in a narrower temperature range than bovine GDH. Half-inactivation temperatures of *C. aceratus* and bovine GDH, calculated after 10 min. of incubation, were 50 and 53°C, respectively.

DISCUSSION

Dissostichus mawsoni and *Chionodraco hamatus* G6PDs, as well as *Chaenocephalus aceratus* GDH, show higher catalytic rates at low temperature than the homologous enzymes from mesophilic sources, in accordance with reports on other enzymes from Antarctic fish (Hochachka and Somero, 1984; Genicot *et al.*, 1988; Somero, 1991) and on cold-adapted enzymes from psychrophilic bacteria (Davail *et al.*, 1994; Feller *et al.*, 1994).

In both cold-adapted and mesophilic organisms, the apparent optimum temperature of G6PD increases up to the point at which irreversible heat inactivation occurs. In contrast, the apparent optimum temperature of *C. aceratus* GDH (25°C) is lower than that of irreversible heat inactivation (above 45°C). This result suggests that, above 25°C, Antarctic GDH, although retaining its overall structure, is only partially active, and that

the formation of the enzyme-substrate and/or enzyme-coenzyme complexes may become less favored.

The effect of exposure to high temperature on the activity of these enzymes indicates that irreversible heat-inactivation occurs at temperatures similar to those measured in mesophilic enzymes and that the presence of small ligands can influence heat inactivation, e.g. as shown by the protecting effect of the allosteric activator AMP on the thermostability of phosphorylase *b*. A common feature shown by these enzymes is worth to be stressed, i.e. heat inactivation occurs very abruptly in a very narrow temperature range. In G6PD and GDH, this temperature range is clearly narrower than that observed for the homologous mesophilic enzymes, suggesting that the molecular structure is highly sensitive to small temperature variations. Although heat inactivation may not have a direct physiological meaning, since it occurs at temperatures far from the physiological ones, it can provide useful information on the molecular structure evolution.

In order to obtain a suitable metabolic flux, a cold-adapted organism needs to rely on a high catalytic efficiency at low temperature. The modifications of enzymes, adopted to reach this goal, seem to be associated with a rather flexible structure, which does not necessarily imply heat inactivation temperatures much lower than those of the mesophilic enzymes but may induce a more subtle alteration of the temperature sensitivity.

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